ANALYTICAL METHOD DEVELOPMENT AND ANALYSIS OF MESQUITOL ABUNDANCE DYNAMICS IN *Prosopis juliflora*

BY

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been presented for any degree course in any other University.

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DEDICATION

This thesis is dedicated to my dear parents Mr. Pius Kita Odero and Mrs. Florence Midega Odero who have supported me all through my education and to Mrs Beatrice Abidha for her financial support.

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ABSTRACT

Several scientific studies reveal that the heartwood of the plant *Prosopis juliflora* contains very high levels of the flavan-3-ol compound (2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,7,8-triol commonly referred to as mesquitol. Mesquitol, exhibits free-radical scavenging properties, antioxidant and α-glucosidase inhibitory properties. This confirms its potential applicability for the treatment of radical oxidative induced diseases like cancer and atherosclerosis. The limit in technology transfer, has made *P. juliflora* a nuisance to people through its invasive nature and the adverse effects of the pods and thorns on livestock especially sheep and goats yet the plant has potent medicinal value. Incidentally, limited research exists on rapid methods for the extraction and quantification of the compound mesquitol. This study therefore aimed at investigating the abundance dynamics of mesquitol and to develop and validate a High Performance Liquid Chromatographic (HPLC) analytical method for quantification of the said compound. Plant samples of different ages were collected from Marigat in Baringo county, during the wet and dry seasons of June and December respectively. Soxhlet and maceration methods of extraction were evaluated for optimality in flavonoids extraction based on their respective percentage yields after which column chromatography was used to isolate mesquitol. Thin layer chromatography (TLC) assisted in pooling together of fractions with similar R_f values while melting point apparatus and Fourier Transform Infrared Spectroscopy were utilized to confirm the isolated flavanol mesquitol. A HPLC-UV analytical method was developed and validated illustrating high accuracy and precision for the quantification of the mesquitol content in different *P. juliflora* samples. The highest percentage yield during extraction was obtained by soxhlet method with the solvent methanol (10.22 ± 2.03 %) for samples collected during the wet season. Total flavonoids however were highest in the acetonic crude extracts (21.7 ±1.1 mg/g equivalent) hence guiding the selection of the extracts for the chromatographic isolation of mesquitol. The purified compound was confirmed to be mesquitol by the characteristic peaks of the flavanols structures which include the O-H broad band absorption at 3350cm^{-1}, the aromatic C=C skeletal vibrations at 1625 cm^{-1}, 1520 $cm^{-1}$and 1480 cm^{-1} which are typical of flavonoids. The melting temperature range was found to be between 82-85°C as previously reported for mesquitol. On quantification, mesquitol was found to be more abundant during the wet seasons for plants of above 4 years age category reaching 642.893 µg/mL. On the other hand, the lowest amount of mesquitol was obtained in the dry season as 181.245 µg/mL. This variations in seasonal abundance, could be attributed to chemo-seasonal dynamics that have been witnessed to affect biosynthesis and deposition of phyto-compounds in plants like temperature, availability of water, humidity etc. The developed and validated HPLC method, illustrated satisfactory quantification of mesquitol at 3-5 % of the crude extract hence illustrating the potential for related applications in agro-food and pharmaceutical industry analysis. Given the age and seasons of harvest are significant it is recommended that evaluation of *P. juliflora* samples of higher age brackets, 12, 16 and 20 years be carried out to establish mesquitol abundance trends to inform exploitation of this valuable natural resource.

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DEFINATION OF TERMS AND ABBREVIATIONS

^{1}H NMR- Proton Nuclear Magnetic Resonance

ASAL- Arid and Semiarid regions.

GC – Gas Chromatography

HPLC- High Performance Liquid Chromatography

ICH- International Council for Harmonization

KEFRI- Kenya Forest Research Institute

LOD- Limit of detection

LOQ- Limit of quantification

MS – Mass Spectroscopy

NIB- National irrigations board

PTLC- Preparative Thin layer chromatography

R_f - Retention factor

SPSS- Statistical Package for Social Sciences

TFC- Total flavonoid content

TLC- Thin layer chromatography

UV-VIS- Ultraviolet visible

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Prosopis juliflora, commonly referred to as the mesquite or the honey mesquite plant in America, is a hardy and woody plant species belonging to the genus *Prosopis* that has about 44 members. In Kenya, the plant is locally referred to as "Mathenge" and it majorly grows in arid and semi-arid areas such as Baringo, Tana-river, Turkana, Kajiado and Garissa counties (Dubow, 2011). The plant, is native to South America, Central America and the Caribbean with 40 of the 44 species being native to both North and South America, and three others originating from Asia and one being native to Africa (Dubow, 2011; Henciya *et al*., 2017).

It is a fast growing, nitrogen fixing and evergreen shrub, that copes easily with a wide variety of environmental and weather conditions, habitats and temperatures (Mwangi and Swallow, 2005). This includes regions that experience great temperature fluctuations and very little rainfall like arid and semi-arid environments (ASAL). It has been noted that given optimum conditions and adequate rainfalls, the tree can grow to heights of up to 18 meters and a girth of up to 1 meter (Alsaadi and Maliki, 2015).

Prosopis juliflora is an invasive plant species and in places where it has been introduced, it has rapidly colonized the environment and destroyed the natural pasture and habitat. This has not only reduced the grazing land and the rangeland that was readily available to livestock, but also in some cases, it has negatively affected the food security of the surrounding communities by colonizing cultivatable land and making it

non-useful. This is also partly because removing the plants totally is very costly (Pasiecznik *et al.*, 2001).

Several studies worldwide have shown that invasive species like *P. juliflora*, are a major cause of loss of biodiversity. This is primarily because, most of them have the natural ability to outdo a majority of the native species by quickly adapting to the environment and colonizing it hence over time competing with them for resources and nutrients and sometimes replacing them (Al – Rawahy *et al.*, 2003).

The plant, was first introduced in Kenya in the late 1970's. This was done by the joint effort of the government of Finland, the National Irrigation Board (NIB) and the Kenya Forestry Research Institute (KEFRI). This was due to the need of the Kenyan government wanting to come up with a permanent solution to curb the then rising levels of deforestation that was driven by the ever increasing need for fuel energy in the forms of charcoal and firewood. This had in turn began causing pressure on the levels of natural forest cover especially in places that were considered as arid and semi-arid as people began to cut down trees (Mwangi and Swallow, 2005).

The plant therefore seemed to be a natural solution because of its natural ability to grow very fast and its resilience to different environmental conditions, including ASAL regions hence it was chosen for afforestation purposes. Initially, the plant was very useful this is because it proved good for the control of soil erosion, the stabilization of sand dunes, the provision of fuel energy recourses both in the forms of charcoal and firewood and for the promotion of honey production. But over the years, the plant began to colonize vast portions of the land, taking control of water points and growing invasively even in lands that were used by communities for arable purposes (Dubow, 2011; Mwangi and Swallow, 2005).

The negative effects of the plant, quickly began to outweigh its positive effects as animals began to feed on it due to its ever green nature even when conditions are dry. This resulted to the loss of gums, facial contortions and disfigurements and sometimes, constipation of the livestock especially goats and cattle. In some rare cases, it also affected the rumen negatively sometimes leading to death of livestock (William and Jafri, 2015).

Despite its negative effects on livestock especially when the pods and leaves are consumed in large quantities, there are reports that the plant has been used as folk remedy for a myriad of diseases in different parts of the world. It has been used for the treatment of common cold, sore throats, different inflammations, dysentery, gonorrhoea, boils, rheumatic pain, digestive problems (Singh, 2012). This can be attributed largely in part to the phytochemical compounds that have been found present in the plants such as tannins, flavonoids, lignans, alkaloids, stilbenes, terpenes, terpenoids and manyothers that are both useful to human beings and to animals. These phytochemicals are not only readily available in different parts of the plant in different concentrations but they have also been proven to have the ability to alter both biological and physiological action in the body hence healing the body. One of the major advantage of medicinal plants over conventional forms of modern synthetic medicine being that they are of lower toxicity and lesser side effects to human beings (Singh, 2012).

Different parts of the plant have been used for the treatment of different ailments for example, the mesquite pollen have been used for their antioxidant abilities, while the flowers have been ground to powder and subsequently used by expectant mothers as a

safeguard against miscarriage. The bark and twigs of the plant have been used for the treatment tooth decay and stomach crumps (Prabha *et al*., 2014).

It is thus because of its potential both as an economic resource and as a source of traditional medicine, that studies on methods to valorise the plant into a more useful form began to be done. In North America, the plant has been used for aesthetic beauty i.e. landscaping, production of furniture, as fence posts and as cattle feed supplements (Pendergrass, 1984). In South American countries like Mexico, the plant has been valorised into edible food products like flour, syrups, beverages and food supplements (Choge *et al.,* 2007). In Peru and Argentina, it has been valorised into biscuits and health tonics (Azam *et al.,* 2011).

Whereas most countries have tried to valorise the plant into a more useful source, most African nations including Kenya, have grossly underutilized the plant using it only for firewood and for the making of charcoal. This is largely because of the lack of technology transfer from the parent countries where the plant originated from. It is for this reason, that most African countries therefore have suffered after the introduction of the plant. Due to this, studies are now ongoing for valorisation of the plant in Kenya (Kingori *et al.,* 2011).

Research done on the phytochemicals present in *P. juliflora* reveals several useful extractives like stilbenes, alkaloids, terpenoids, flavonoids, tannins and steroids among others which are well distributed in the plant (Lakshimbai *et al*., 2015). In his research, Sirmah (2009) noted that the flav-3-ol compound (2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,7,8-triol commonly referred to as mesquitol, was particularly abundant in unusually high concentrations at the heartwood of the plant with lesser quantities in the sapwood, gum and bark of the plant.

It has been noted that the amount of plant metabolites present in plants generally vary as a result of several biological, environmental and physiological factors i.e UV radiations, seasons, weather, temperature, altitudes, nutrients among many other things (Vagiri *et al*. 2015). Chen (1981) noted that the total amount of organic material extracted from *P. juliflora* varied as a function of the season of harvest, specifically noting that the amount of extractives harvested in the winter season were the least with the spring season being the highest among the four seasons.

Further research work on the same, was done by Pendergrass (1984) specifically testing for the seasonal variations of the levels of non-carbonate organic extracts over a period of two years with different seasons and found that the highest yields, the same, was during the late spring and early summer periods. This results, were tallying well with Chens results suggesting that the winter period was the worst time to harness plant extractives from *P. juliflora* (Chen 1981; Pendergrass, 1984).

Some countries like America have an annual cycle of four seasons i.e. autumn, summer, spring and winter, most tropical countries have only have two distinct seasons i.e. dry seasons and wet seasons. In Marigat Baringo county were the plant samples were harvested from, the wet seasons, are characterized by lower temperatures and plenty of rainfalls while the dry seasons are characterized by elevated temperatures and little or no rainfall (Lelenguyah *et al.,* 2016).

Whereas data exists on the seasonal variations of non-carbohydrate organic compounds at large from *P. juliflora*, in areas with the four said annual cycles, little data exists on the seasonal abundance of non-carbonate organic compounds and flavanols like mesquitol in arid and semi-arid tropical areas including Baringo county over the wet and dry seasons. Therefore, this research was aimed at bridging the gap by generating

data specifically on the total flavonoid content of the *P. juliflora* plant, the mesquitol abundance during the two seasons experienced in Marigat Baringo County and to compare mesquitol content in plants of different ages.

1.2 Statement of the Problem

The lack of transfer of technology, on how to valorise the *P. juliflora* plant from its countries of origin, has made it a nuisance to a majority of the people especially those in 3rd world countries. This is because of its invasive nature and the effects of the pods on livestock especially sheep and goats which lose their teeth and in some cases die (Choge *et al.,* 2007).

Amidst all these disadvantages, the plant was discovered to have many important phytochemicals that are of great medicinal importance like alkaloids, stilbenes, flavonoids etc (Singh 2012). Sirmah (2009) noted that the heartwood of the plant has an unusually high levels of mesquitol. Mesquitol is a plant extractive that belongs to a group of flavan-3-ol compounds that are known to have great antioxidant and radical scavenging properties (Rao *et al.,* 2003). This means that mesquitol can be used for the treatment and recovery of many radically oxidative induced diseases like cancer and atherosclerosis due to the fact that it is able to stimulate the endogenous body defence system (Yamaguchi *et al*.,1998).

Despite its natural presence, abundance and its illustration of great potency both in the pharmaceutical and agro-food industry as both medicine and food supplements respectively, limited research exists on rapid methods for the extraction and quantification and of the compound mesquitol. This study therefore was out to provide important information on mesquitol abundance dynamics during the wet and the dry

seasons hence providing future useful information for mesquitol natural resource valorisation.

1.3 Objectives

1.3.1 General Objective

The main objective of the study was to evaluate and quantify the flavonoid mesquitol content variations in the plant *Prosopis juliflora* as a function of season and age.

1.3.2 Specific Objectives

The specific objectives of this study were to:

i) Optimize an extraction scheme for flavonoids and to determine the total flavonoid content of the heartwood extracts from *P. juliflora* plant.

ii) Extract and isolate mesquitol from heartwood extracts of *P. juliflora* plant

iii) Develop and validate a method for the quantification of mesquitol from *P. juliflora.*

iv) Evaluate the variations of mesquitol content in *P. juliflora* plant based on their ages and seasons of harvest.

1.4 Hypotheses of the Study

For the objective number (iv) of the study to be achieved and satisfied, three hypotheses were formulated and tested at significance level of 0.05.

Null hypothesis 1 (H_{o1}) the amount of mesquitol in *P. juliflora* does not vary as a result of the season of harvest.

Null hypothesis 2 (H_{o2}) the amount of mesquitol in *P. juliflora* does not vary as a result of the age of the plant.

Null hypothesis 3 (H_{o3}) the amount of mesquitol in *P. juliflora* does not vary as a result of both the age and season of harvest.

1.5 Justification

Recent studies done on the flavanol group, indicate that they have great therapeutic potential. This has been illustrated especially when it comes to the management and treatment of inflammatory diseases and conditions including diabetes, cancer and a variety of other diseases (Rao *et al*., 2003; Maobe, 2013).

Meanwhile several studies revealed that the heartwood of the plant *P. juliflora* contains very high level amounts of the flavan-3-ol compound mesquitol (Sirmah *et al.,* 2011). Mesquitol, like other flavanols, is reported to have antioxidant properties, exhibit free-radical scavenging properties and α-glucosidase inhibitory properties hence it is thought to be useful as a form of medicine (Rao *et al*., 2003).

Currently, a vast majority of pharmaceutical research has shifted from synthetic forms of medicine to herbal medicines by the extraction and analysis of plants that are perceived to be of medicinal value in what is referred to as the "Return to Nature" research. This is majorly due to the fact that most of them are less toxic when compared to conventional medicine and also because they have limited side effects (Dave and Bhandari, 2013). A major advantage also being that, medicinal plants possess important biologically active compounds that can be extracted, isolated, examined and structurally determined. This means that they can be later used as precursors for formulation of modern medicine (William and Jafri, 2015).

Laboratory experiments done on mesquitol, have shown that unlike many other known flavonoids, that have antioxidant properties only, it has high levels of radical scavenging properties and α-glucosidase inhibitory properties (Rao *et al*., 2003). Research work done by Azam and others (2011) compared the strengths of mesquitol with common antioxidants currently in the market and used as medicine like probucol and α-tocopherol and found that mesquitol was a stronger antioxidant than both. This indicates its great potential for use as medicine and food supplements. This study, will thus be useful in the quantification of mesquitol content in different seasons and age of the *P. juliflora* plant.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of the Plant *Prosois juliflora*

World over, different plants species have been imported to different countries for various reasons including their economic, medicinal, environmental and in some cases aesthetic importance. *Prosopis juliflora* is a hardy, invasive plant that is native to parts of both South and North America where it is also commonly referred to as either honey mesquite, the mesquite plant or algarrobo. The term hardy, is used to describe it because the plant is tolerant to drought, water logging, alkalinity, salinity and a myriad of other environmental conditions (Dubow, 2011).

P. juliflora is a shrub that can grow to heights of up to 18 meters with trunks of up to 1 meter if grown in areas of favourable conditions, with a tap root system that can penetrate deep into the soil in search of nutrients and water (Alsaadi and Al-Maliki, 2015). They are commonly referred to as xerophytic plants that is, an evergreen shrub that easily adapts well to a wide variety of different soil conditions and types of climates (Mwangi and Swallow, 2005). The plant, can survive in very harsh environments including regions that are known to have scarce rainfalls of around 500 mm annually and temperature fluctuations of between -12 to 50 degrees celsius like arid and semi- arid regions (Mwangi and Swallow, 2005; William and Jafri, 2015).

The plant was first introduced to Africa in Senegal in the year 1822 and subsequently in other countries where it was thought to be useful over a period of time. Presently, the plant exists in more than twenty-five countries of Africa including Kenya (Mwangi and Swallow, 2005). In Ethiopia, the plant was introduced by the government

for use as timber, forage, food and medicine where as in Kenya, it was introduced by the joint effort of the Kenya Forest Research Institute (KEFRI) the government of the People of Finland and the National Irrigations board of Kenya in the late 1970's. This, was done with the aim of getting a solution to the level of deforestation that was affecting the country at that point in time and to provide an alternative method of harvesting of charcoal and fuel energy (firewood) especially in regions that where considered to be arid and semiarid (Mwangi and Swallow, 2005; Aboud *et al.,* 2005). Because of the plants ability to grow very fast and its resilience to variant environmental conditions, the plant proved to be good for the control of soil erosion, reforestation of ASAL regions and the provision of fuel energy especially in the form of charcoal and firewood. But over the years, the disadvantages of the tree began to manifest as it began to colonize vast portions of grazing and arable land taking control of crucial water points resulting to the loss of useful land and water sources (Mwangi and Swallow, 2005).

In developed nations and economies, the tree is a source of valuable multipurpose products, and all the parts of the plant are considered as useful. The trunk of the tree, is used for the making of timber, building poles, furniture, charcoal and firewood. The pods, are also used for fodder and making of flour, biscuits and other beverages (Van Den Eynden *et al.,* 2003; William and Jafri, 2015). In some parts of Mexico, local economies have been built solely based on the plant and its products. For example, the pods are stored as fodder or they are transformed into nutritious syrups that are locally sold in the supermarkets as human food. The consumption of products derived from *P. juliflora* by humans as a source of nutrition has been considered safe in many nations including nations where the plant is used as staple food. So far, food poisoning from food products made from *P. juliflora* has never been recorded especially when it is

consumed after being cooked. In fact, flour that has been generated from the seeds of *P. juliflora* in Argentina, have been tested in the lab for toxicity and aflatoxins and found to be not only very nutritious but also fit for human consumption (Choge *et al.,* 2007)

Despite the many uses of the tree, in almost every region that the tree has been introduced, it is reported to be a very invasive species. In fact, the Invasive Species Specialist Group of the IUCN voted for the plant as one of the most unwanted species of plants in the world in the year 2004. The tree does not only invade the region, it colonizes it. This is done by placing a high demand on water and nutrients in the soil and in turn depriving other plants that are next to it. This has affected regions that are considered grazing field leading to a situation where the animals are made to feed solely on *Prosopis* leaves. (Mwangi and Swallow, 2005). Figure 2.1 below shows the *P. juliflora* plant.

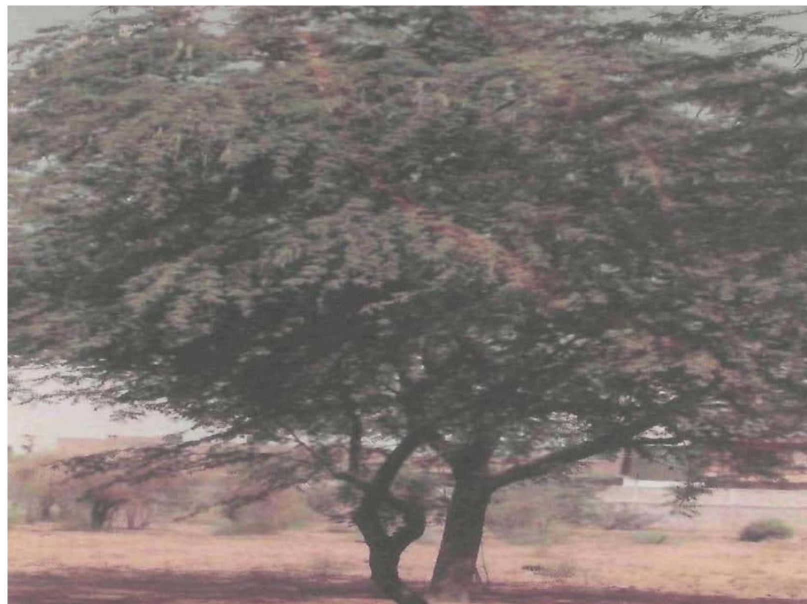


Figure 2.1 the *P. juliflora* plant (Source: Azam *et al.*, 2011)

2.2 Plant extractives of *Prosopis juliflora*

The term plant extractives is generally used to refer to a group of compounds that are of low molecular weight and that are naturally present in a plant in significant quantities alongside hemicellulose, lignin and cellulose (Chang *et al.,* 2001). These extractives are obtainable easily from the plant by simple extraction of the dry grounded plant material by simple use of organic solvents of different polarities e.g. hexane, dichloromethane, acetone, ethanol, methanol or water through various methods like soxhlet extraction, steam distillation, dionex extraction or any other suitable extraction method (Toshiaki, 2001).

The plant extractives, vary greatly from plant to plant and from species to species thus providing a tremendous reservoir of various compounds and chemical substances with great therapeutic effects and purposes. This is because, most of this plant extractives serve as the defence systems for the plant based on its environment and attacks from pests and animals hence they are continuously being synthesized (Adebanjo and Adewumi, 1983). Pharmacological research evidently shows that plants with desired plant extractives can be valuable as both food and beverage additives (Harborne and Williams, 2000). It has been noted that, plants generally contain variants of these extractives that are different in nature, use and importance to the human body and to animals both as medicine and in some cases as poison due to their toxicity.

Several biological and pharmaceutical researches show that many of these plant extractives to possess important medicinal values. This is largely because they can alter certain physiological and biological actions in the human body with the most important of this compounds being terpenes, alkaloids, stilbenes, flavonoids and phenolic compounds (Alsaadi and Al-Maliki, 2015).

Flavonoids for example, show antioxidant, anti-inflammatory and anticancer properties and for this reason, plants that contain it are used for treatment of cancers and heart related ailments. On the other hand, compounds like alkaloids have antibacterial and analgesic properties, making plants good sources of herbal remedies (Ibrahim *et al.,* 2013). They are thus of great use especially as sources of crude medicines even for domestic animals.

Despite most medicinal plants having plant extractives in them, it has been noted that in most cases the extractives are not evenly distributed within the plant with some parts having more extractives than others. It has been noted that, in most plant species, plant extractives are in plenty at the heartwood and lesser in quantities, at the sap wood, bark, seeds, roots and leaves. It has also been noted that in most temperate regions, although plant extractives vary in seasons and in quantity, they generally have a percentage abundance of between 5 % - 14 % (Toshiaki, 2001).

Previous research done on *P. juliflora*, shows that it is a very rich source of natural extractives with several phytochemicals like flavonoids, alkanoids, phenols, terpenes, tannins, steroids, phenolic and cardiac glycoside which are all medicinal in nature present (Singh, 2012; Lakshimbai *et al.,* 2015). The following are examples of naturally occurring phytochemicals in the medicinal plants and more specifically the *P. juliflora* plant.

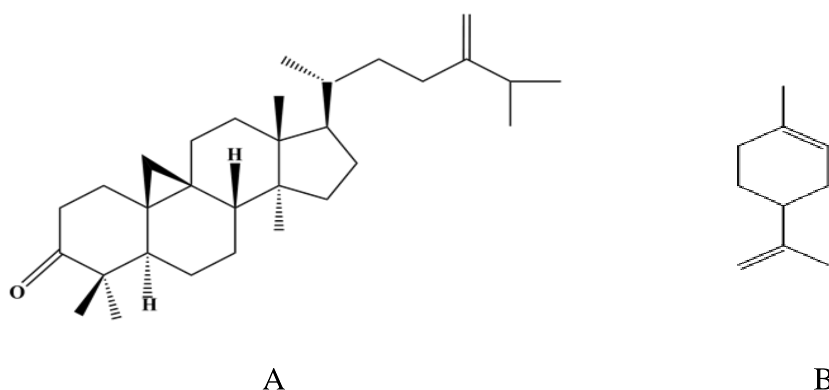
2.2.1 Terpenes and Terpenoids

Terpenes refer to a group of fragrant volatile unsaturated hydrocarbons that are oily in nature. Their structures are based on the isoprene structure hence they have protective functions, this makes them useful especially for insecticides (Singh 2012). A phytochemical analysis of the *P. juliflora* plant done by Singh (2012), showed

terpenes to be present in very high concentrations both in the leaves and pods of the plant and in moderate and low concentrations at the flowers and roots respectively. Terpenoids on the other hand, are simply terpenes that have undergone a chemical modification as a result of either an oxidation process or a rearrangement process on the carbon structure.

Research done by Alsaadi and Al-Maliki (2015), showed the presence of terpenoids in the pods of the plant. They managed to isolate the terpenoids 24-methylencycloartan-3-one. Further studies on this compound, showed that it has hypo-glycaemic effects on the body hence it can effectively be used for the treatment and management of diabetes mellitus (hypoglycaemia) instead of insulin with no cytotoxicity at all to human beings.

Figure 2.2 shows the terpenoid 24-methylencycloartan-3- one and the terpene: limonene.



A B

Figure 2.2 A- 24-methylencycloartan-3- one and B- Limonene

2.2.2 Tannins

Tannins refer to a group of phenolic compounds in some plants that are able to convert or change animal skin to useful leather in a process referred to as tannin. They are colourless and non-crystalline compounds widely distributed in the plants with the ability to dissolve in water and precipitate alkaloids, gelatin and other protein due to the

presence of both the carboxylic and phenolic groups within its structure (Trease and Evans, 1989; Toshiaki, 2001).

Tannins have been used as antiseptic, antimicrobial agents, antioxidants with radical scavenging properties etc. Phytochemical analysis done by Sachi Singh (2012), showed tannin to be present in *P. juliflora* although in relatively low concentrations at the leaf, pods and roots. Ibrahim *et al.,* (2013) also found tannins to be present in the leaves of the plant although in low concentrations. Tannins are classified into two broad categories of hydrolysable and condensed tannins. Figure 2.3 below shows an example of condensed tannins an oligomer of polyhydroxyflavan-3-o1 unit.

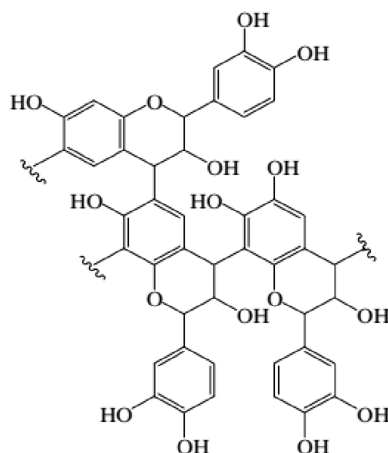


Figure 2.3 Condensed tannins

2.2.3 Alkaloids

Alkaloids are cyclic organic compounds containing nitrogen in a negative state of oxidation. Most are basic hence the name alkaloids which is derived from the word alkali. They include drugs like nicotine, morphine and quinine and poisons like atropine. Alkaloids are the most important of all plant extractive groups, and they comprise of more than 4000 known types of alkaloids that have been extracted, isolated and characterized from plants. In as far as manufacturing of drugs based on plants has been done, alkaloids have been the basis of some of the most important and useful drugs

today like morphine which has been used as pain killers and quinine which has been used as anti-malarials (Trease and Evans, 1989; Toshiaki, 2001).

Alkaloids have been shown to possess a wide variety of medicinal properties for example, morphine has been used an antidepressant while cocaine has been used as an anaesthetic, caffeine as a stimulant, cordeine as a cough expectorant, hernine as an analgesic and berbine as an antibacterial (Heinrichn *et al.*, 2004). Several alkaloids with important, pharmacological activities have been isolated from the plant *Prosopis juliflora*. They include juliflorine, seco-juliprosopinal, juliprosine, isojuliprosine, which are known to have antioxidant, antifungal, and anti-bacterial properties (Ibrahim *et al.,* 2013). Figure 2.4 below shows an example of the alkaloid juliprosine found in *P. juliflora*.

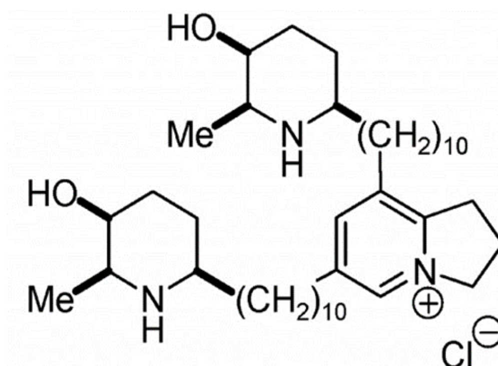


Figure 2.4 juliprosine

2.2.4 Saponins

Saponins, occur primarily within the kingdom plantae, but they can be found distributed widely in nature. The term was derived from the plant *Saponaria vaccaria* which had soap like characteristics due to the prescence of Saponins. Therefore, most saponins, have soap like characteristics as they lower the surface tensions of most aqueous solutions. Saponins are generally poisonous especially to cattle as they cause

haemolysis of blood (Kar, 2007). Figure 2.5 shows the structure of the compound solanine which is an example of saponin.

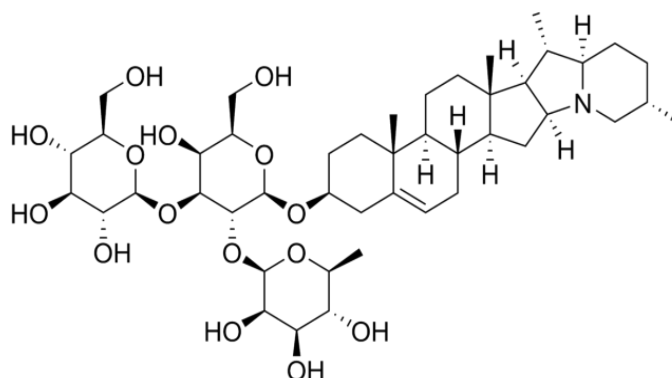


Figure 2.5: The Structure of the Saponin Solanine

2.2.5 Flavonoids

Flavonoids are a group of phyto-nutrients that widely exist naturally in most fruits and vegetables. Structurally, flavonoids are composed of a 15-carbon skeleton consisting of two phenyl rings and a heterocyclic ring. They occur in large quantities at the bark, heartwood and leaves and in lesser amounts on the fruits, flowers and other parts of the plant *Prosopis* (Ibrahim *et al*., 2013).

They are categorized into iso-flavonoids, flavan-3-ols, flavanones, (leucoanthocyanidins), neoflavanoids, aurones and dihydroxylavanols with most flavonoids being waters soluble and more than 4000 variants having so far been isolated and characterized from different plant species (Sirmah, 2009; Maobe, 2013).

They are scientifically proven to be physiologically active hence they are used as medicine for their anticancer, antivirus and anti-inflammatory effects (Maobe, 2013). Flavonoids are responsible for protecting the plant from ultraviolet rays and for the diversity of color in the heartwood of different plants. Some flavonols like 3,4,7,8 tetrahydroxyflavone show anti-termitic properties and are therefore thought to have a

great effect on the durability on the woody parts of the plant while others, have inhibitory activities against organisms that cause diseases in plants (Sirmah, 2009).

A number of flavonoids have been isolated from the *P. juliflora* plant and they include catechin, mesquitols, epi-catechin, 6 flavones--apigenin, luteolin, quercetin 3-O-diglycoside etc (Dave and Bhandari, 2013). Figure 2.6 shows an example of a flavonoid compound

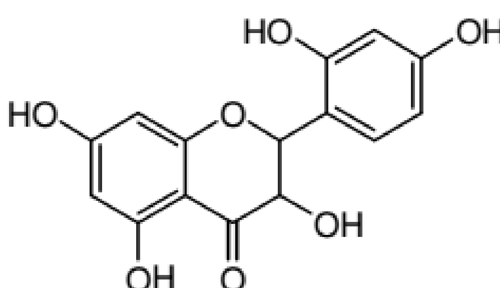


Figure 2.6: An example of a flavanoid compound

2.2.5.1 Catechin and mesquitol

Mesquitol and catechin both belong to the group flavan-3-ol which are the most common of all groups of flavonoids. They are known to have a lot of biological effects like being very strong antioxidants, being anticarcinogenic and antiobesity (Aiyelagbe and Osamudiamen, 2009).

Structurally they look alike with minor differences in the positions of the OH group. Catechin, is naturally present in wine, fruits, cocoa, tea and some fruits with studies showing that catechins have physiological properties especially anti oxidative and anticancer effects (Moridani *et al.,* 2001). Small amounts of catechin and epichatechin have been found to be present in the heartwood of the plant *P. juliflora*. Figure 2.7 below shows the structure of catechin.

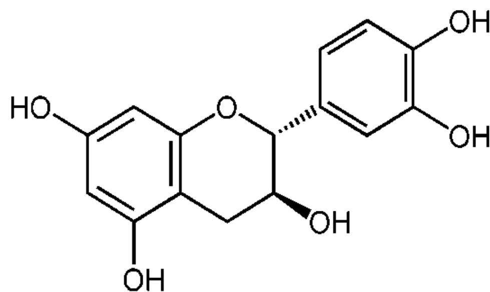


Figure 2.7: Structure of Catechin

Mesquitol on the other hand is also an antioxidant from the flavan-3-ol group whose isomer has been isolated in excellent yields from the *Dichrostachys cinerea* plant. Its scientifically referred to as (2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,7,8-triol by the IUPAC. Work done by Rao *et al.*, (2003) showed that mesquitol is an antioxidant with radical scavenging properties and α-glucosidase inhibitory properties meaning that, they are able to stimulate the endogenous body defence system hence they are capable of treating and preventing diseases like cancers and diabetes hence, its potential use both as herbal remedies or as additives in food.

Azam *et al.,* (2011) compared the antioxidant potential of mesquitol with two commonly used drugs in the market i.e probucol and α-tocopherol and found mesquitol to much stronger than both. Research done by (Suresh *et al.*, 2012) shows that mesquitol has great potential for use as a lead natural organic compound that can be used for future development of anti-glycating agents with potent antioxidant properties. Sirmah *et al.*, (2011) found mesquitol to be abundant in the heartwood of the *P. juliflora* plant. Figure 2.8 shows the structure of mesquitol.

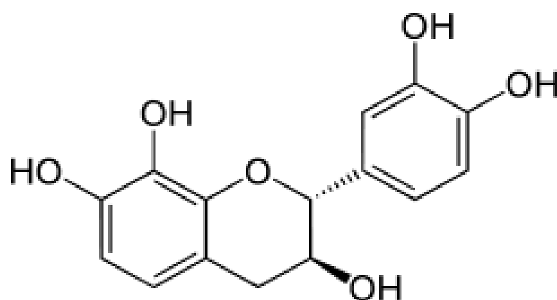


Figure 2.8: Structure of Mesquitol

2.3 Choice of Solvents and Extraction Method

For any extraction process of biologically active compounds to be successful, it largely depends on the type of solvent used and the technique of extraction. Several properties, make a solvent good for extraction purposes and they include the ability to retain the integrity of the extract i.e it must not form complexes or make it dissociate, it should be of low toxicity and it should easily evaporate (Das *et al.,* 2010).

Azusanida (2015) noted that there is no universal extraction method that is ideal or that can be considered as the best extraction technique. This is because, each extraction technique, is unique to different plants and to different extractives of interest. It is for this reason, that this project will compare two commonly used extraction techniques namely soxhlet and maceration and four solvents of different polarities i.e hexane, dichloromethane, acetone and methanol.

2.4 Isolation, identification and quantification of Mesquitol

2.4.1 Chromatographic isolation of mesquitol

Chromatography, is a method of separation of mixtures that employs the use of a mobile phase and a stationery phase that are immiscible in nature. Chromatography

employs the interactions between the mobile phase which is either a liquid or gas, and the stationery phase based on their differences in their physico-chemical properties like size, mass, volume, charge, binding interaction etc (Joshi, 2012). In cases where there are mixtures e.g. in crude extracts of plants, then column chromatography and TLC are both employed for separation of the said phytochemicals.

2.4.2 Thin Layer Chromatography and Preparative Thin Layer Chromatography

The TLC represents one of the fastest, reliable and fairly inexpensive methods of identification that gives a rough idea as to how may components are present in a mixture of many compounds. The TLC is qualitative in nature in that, it is used for the identification of different compounds in a mixture by comparison of R_f values with those of known compounds when run under identical conditions (Patil and Shettigar 2010; Jagessar *et al*., 2007). Figure 2.9 shows an example of a TLC plate

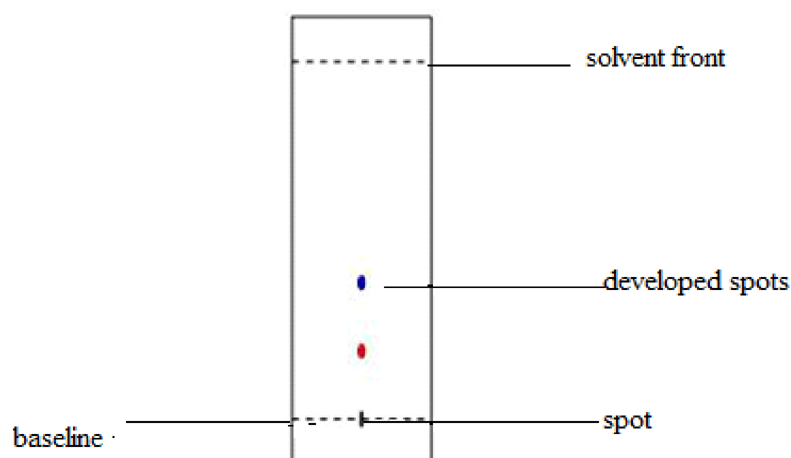


Figure 2.9: An Example of a TLC Plate

It has been used in the profiling of active ingredients of medicinal plants for alkaloids, tannins, flavonoids, saponins. The TLC, is usually spotted at the base on the stationery phase and placed on a mobile phase then left to develop. Because of the variant

solubilities and strength of their adsorption on the stationery phase the plate will show the various compounds presents. On reaching the desired solvent front, the visible compounds are observed by the eye and the invisible compounds visualized with either a UV lamp or by spraying (Sasidharan *et al*., 2011).

Preparative TLC on the other hand, are used for analytical isolations of fairly larger quantities of material usually 10-1000 mg. Samples are loaded at the base like in the case of TLC although they are made in a straight line at the base and left to develop.

2.4.3 Identification of mesquitol

The structure of mesquitol can be established using a mixture of various physical and spectroscopic methods (*Rao et al.,* 2003). Sirmah (2009) characterized and quantified mesquitol by the use of ^{1}H NMR (Proton nuclear magnetic resonance), GC-MS (gas chromatography coupled with a mass spectrophotometer), FT-IR (Fourier Transformation Infra-Red) and HPLC-UV (High Performance Liquid Chromatography coupled with a UV detector).

2.4.4 HPLC quantification of mesquitol

Margari and Tsabolatidou, (2015) noted that for the qualitative and the quantitative determination of organic compounds and phytochemicals, either the GC or the HPLC can be used. However, they also noted that because of the lack of volatility of most phenolic compounds, the GC is an unsuitable choice for the quantification of most phenolic compound although it is still used sometimes. This is because, for them to be quantified effectively in a GC, derivatization must be done. It is for this reason then that the HPLC coupled with a suitable detector, represents the most reliable and the most preferred technique used for the quantification of

phenolic compounds. Quantification of phenolics and flavanoids in HPLC are usually done using commercial standards especially when they are available (Margari and Tsabolatidou, 2015).

2.5 Seasonal Variations of Plant Extractives

Gouvea *et al.,* (2012), noted that despite the existence of gene expression, genetic control and genotypes, the amount of plant extractives found within a plant greatly vary as a result of season, availability of water, temperature, U.V light, altitude, availability of nutrients, environment, the stage of growth etc (Vagiri *et al*. 2015).

This is because the process of the production of this plant metabolites, is largely as a result of its adaptive process of the plant in response to external factors surrounding it. Because of this, it has been proven that plant metabolites are never the same even within the same species as they will vary over time, seasons, and even geographical locations (Koleva *et al.,* 2002).

Several studies published in reputable scientific journals, indicate that there exists no general rule as to when it is best to harvest plant extractives as they vary from one plant species to another. For example, it has been noted that in some plant species, the summer period is the best to harvest phytochemicals as they are in plenty with the yields, drastically decreasing as you approach the winter period. While in some other species, it is vice versa i.e the yield drastically increases as you tend towards winter (Soni *et al.,* 2015). It is therefore of great importance to therefore first study the amount of phytochemicals present in a season before delving to exploit them commercially for maximum profits and reduction of wastage (Nascimento *et al*., 2015).

In his research, Chen (1981) studied the abundance dynamics of polar and non-polar fractions of *P. juliflora* over an extended period of one year. He concluded that they can best be harvested during the spring period. He equally found that there was a drastic reduction in the levels of phytochemicals as winter approached and a subsequent rise as summer sets in. His results, were also confirmed by Pendergrass (1984) who also did a study on the abundance dynamics of non-carbohydrate organic compounds of *P. juliflora* over a period of two years. His results mirrored those of Chen with his results showing that non carbohydrate organic compounds were most plenty during the late spring and early summer seasons.

Whereas, in some countries seasons like winter and summer exist, most tropical regions like Kenya, neither have summer nor winter does but only two distinct seasons i.e dry season and wet seasons which are defined by a difference in the amounts of rainfalls experienced.

2.6 Method Development and Validation

Analytical methods development, refers to the coming up with a method that can be used for the quantitative or qualitative determinations of a sample accurately and reliably. On the other hand, method validation refers to the process that is used to confirm the suitability of an analytical procedure (Kalra, 2011).

The aim of method development and validation is to ensure that the results that are obtained are reliable, consistent and accurate. Validation is usually done according to ICH guidelines (ICH, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials and Reagents/ Chemicals

3.1.1 Chemicals

With the exception of hexane, dichloromethane, acetone and methanol that were of regular grade and initially used solely for the purposes of extraction of crude extracts, all the other respective chemicals and reagents used in this study, were of the analytical grade, manufactured by Sigma Aldrich Company and sourced and supplied by Kobian Laboratory supplies and gel sap laboratories, Nairobi. They include, sulphuric acid, potassium iodide, quercetin, aluminium chloride, hydrochloric acid, ethyl acetate and silica gel. Pure mesquitol, was a donation from Dr. Were L.L. Munyendo through the University of Lorraine – Moi University joint project. It was sourced from LERMAB laboratories at the Nancy University (France).

3.1.2 Equipment and glassware

The various equipment that were used to carry out experimental procedures included blender (ramtons), analytical balance, water bath, rotary evaporator (BÜCHI Rota vapor R-114), melting point apparatus (XT4-100B), glass Columns, Test tubes, Capillary tube, 50 mL burette, 1 mL pipette, 10 mL dropping pipette, electric oven, 250 mL conical flask , Thin Layer Chromatography plates (Merck Company), HPLC (Shimadzu LC–10AT Liquid Chromatography system with SPD–10A UV-Vis detector) , FT-IR (Shimadzu FT-IR-8400) and UV- 1800 Shimadzu.

3.1.3 Plant collection, identification and coding

The plant samples of *Prosopis juliflora* were collected from Marigat in Baringo county (latitude 0°, 28’ 0.01” N, longitude 35°, 57’ 0.01 E).

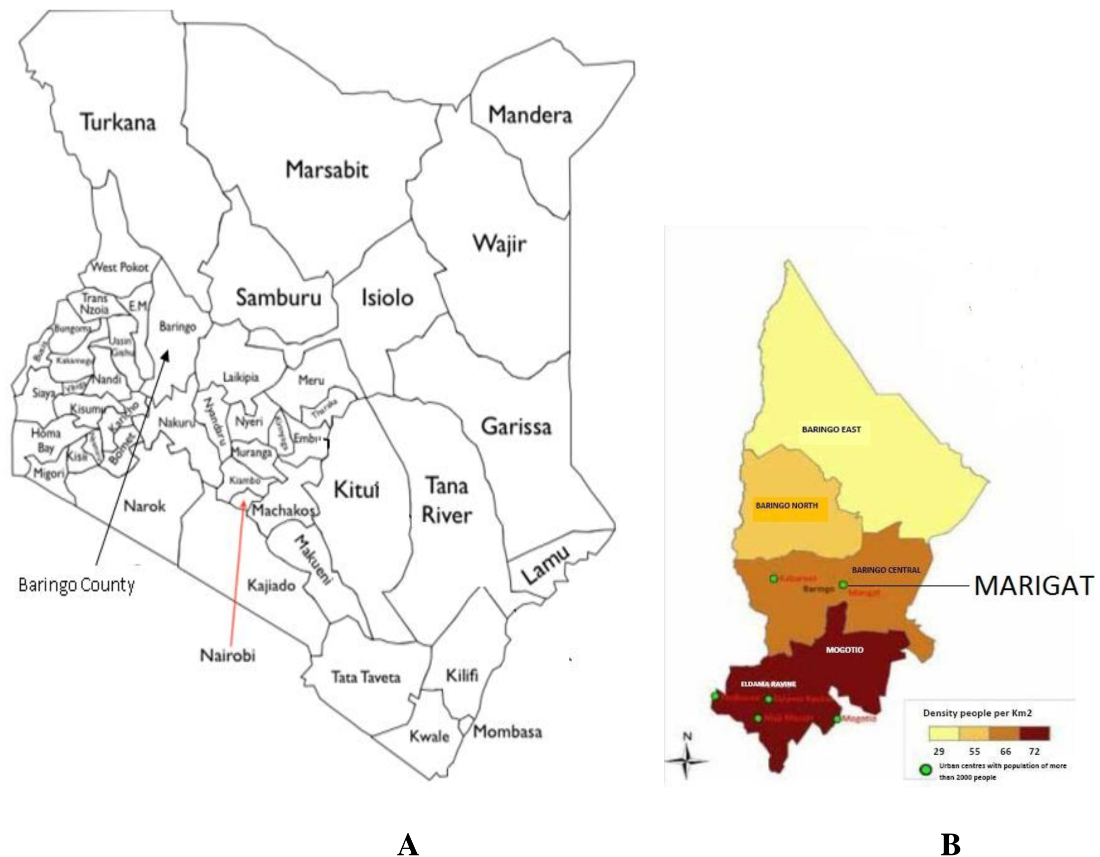


Figure 3.1: A Map of the republic of Kenya showing the location of Baringo County and B Map of Baringo County showing the location of Marigat.

http://softkenya.com/map/kenya-county-map.

The collected plant samples, were grouped into two categories based on their age brackets as determined both by physical observations and information obtained from the local villagers. The first category, entailed plants deemed to be below four (4) years of age while the other category, comprised of those regarded to be above the age of four (4) years.

A second separation was also made with respect to the sampling season, that is either the wet or dry season. The wet season was in the month of June and dry season in the months of December 2016. The harvested plant materials were appropriately cut using a power saw and transported to the Chemistry laboratory of Moi University at main campus. Plant identification was done by Mr. Taabu Tepeny of the Department of Botany, School of Biological and Physical Sciences, and voucher specimen deposited appropriately at the department with accession number assigned as MU/BL/0040/2016.

Sample codes, were subsequently assigned for each of the samples as shown in table 3.1 and henceforth used as unique identifiers for each of the samples used.

Table 3.1: Sample Codes as Unique Identifiers

Sampling season	Plant age bracket	Sample unique identifier
Wet season	Below four (4) years	WBl
	Above four (4) years	WAb
Dry season	Below four (4) years	DBl
	Above four (4) years	Dab

Key: WBl: Plant of below 4 years of age harvested during the wet season.
WAb: Plant of above 4 years of age harvested during the wet season.
DBl: Plant of below 4 years of age harvested during the dry season
DAb: Plant of above 4 years of age harvested during the dry season.

3.2 Methods

All the glass wares that were used in this study were thoroughly washed with water and detergent then subsequently rinsed with distilled water and acetone. They were later dried in the oven at 105°C over a period of approximately 4 hours. All chemicals and reagents that were of regular grade were first before being used for extraction.

3.2.1 Sample processing

Based on previous studies by Sirmah *et al*., (2011) the heart wood of the plant was selected for this study. Freshly cut plant logs were weighed before being cut into small pieces and being air dried in the laboratory.

The dried plant material was then ground into fine powder using a locally assembled grinder (known as a posho mill) and further blended, to increase the surface area to volume ratios of the sample and to enhance the contact between the solvent and the sample during the extraction process.

3.2.2 Solvent extraction

Two different methods of extractions namely maceration and soxhlet method of extraction were initially used in this study for the purposes of comparison. The most effective extraction method was then chosen for subsequent extractions.

3.2.2.1 Soxhlet extraction

The solvents hexane, dichloromethane, acetone and methanol were used in the serial extraction of the fine ground powder. This was done by successively extracting an initial amount of 25 gms of the heartwood, with 250 mL of the respective solvents increasing the polarity simultaneously from hexane to dichloromethane to acetone and

finally to methanol. The solvents with the respective extractives were then rota vaporised to get the amount of extract obtained in each solvent.

3.2.2.2 Maceration technique

On the other hand, for the maceration method 50 grams of heartwood powder was soaked in 500 mL of solvent for up to 48 hours with physical agitations at room temperature in a conical flask. After this, the mixture was filtered with whatman filer paper number 1. The plant extract that had been collected by the filter paper was then re-extracted serially using a more polar solvent with the excess solvent collected after filtration being rota evaporated. This was done stepwise starting with hexane then dichloromethane then acetone then methanol.

3.2.2.3 Assessment of the effect of solvent blends on extractive contents

Different solvents and solvent blends were subsequently evaluated for their suitability for extraction by comparing their respective percentage yields to establish an optimum extraction scheme. This was done by the blending of the four solvents in the ratios of 1:1 i.e hexane:dichloromethane, hexane:methanol, hexane: acetone, dichloromethane:acetone, dichloromethane;methanol and acetone:methanol. A mass of 25 grams of the plant material was then extracted in a soxhlet apparatus each using the blends and the results calculated.

3.2.3 Flavonoids extraction scheme optimization

3.2.3.1 Quantification of total crude extractives

The total mass of each of the extracts of hexane, dichloromethane, acetone and methanol were then obtained by calculating the differences between the measured

masses of the bottles plus the contents in it and that of the bottles when they are empty. The most desirable solvent were those that had the highest mass recovery. The percentage yields were then calculated according to the formula below in equation 3.1.

$$\% \text{ Yield} = \frac{(\text{mass of extract})}{(\text{mass of sample})} \times 100 \text{ .... equation 3.1}$$

3.2.3.2 Determination of Total flavonoid content

Based on Sirmahs *et al.,* (2009) study that showed the prescence of flavonoids, total flavonoid content was done on the plants heartwood. The extracts from hexane, dichloromethane, acetone, methanol and the said solvent blends was tested for TFC by the employment of the Aluminium Chloride colorimetric method as described by Hossain *et al.,* (2013) with little modifications.

This was done by taking 0.25 grams each of the extracts and dissolving them with 1.25 mL of distilled water and approximately 75 µL and of 6 % of $NaNO_3$ was added and the solution thoroughly mixed. The mixture was then incubated in a dark cabinet for approximately 6 minutes then 10 % $AlCl_3$ (150 µL) added to each of the test tubes and subsequently incubated again for approximately 5 minutes in a dark cabinet.

A standard calibration curve was obtained by using quercetin of concentrations 0, 2.5, 10, 20, 40, 80 µg / mL diluted in methanol. This was then used for measuring of absorbance at a wavelength of λ_{max} 510 nm using a UV-Vis spectrophotometer. This were done in triplicates and subsequent results averaged. The total flavonoid content was calculated using formula 3.2 below

$$\text{TFC}=\frac{\text{Absorbance Of crude extracts} \times \text{Mass of quercetin in mg}}{\text{Absorbance of standard} \times \text{mass of extracts in mg}} \text{ .... Equation 3.2}$$

3.3 Isolation of the flavonoid mesquitol from *P. juliflora* extract

3.3.1 Column Chromatographic isolation

The resultant crude extracts with the highest total flavonoid contents (TFC), were subjected to column chromatography for the isolation of the compound mesquitol. 75 mg of crude acetone extract from each of the *P. juliflora* four samples (WBl-Ace, WAb-Ace, DBl-Ace and DAb-Ace) were adsorbed onto 100 grams of silica gel (S 0.032-0.063mm) each separately packed into a glass column of internal diameter 2.6 cm, external diameter 3cm and a height of approximately 66 cm.

Normal phase chromatographic gradient elution started with 10 mL of a mixture of hexane to DCM in the ratios of 9:1 then 8:2 systematically changing the ratios of the reagents upto 1:9 each time collecting the eluates in test tubes. The same sample was then eluted with DCM: EtOAc starting with ratios 9:1 equally changing the ratios systematically downwards upto to 1:9. This was repeated again with EtOAc:Ace in the ratios of 9:1 up to 1:9 each time the eluents being collected in test tubes in portions of 5 mLs each.

3.3.2 Thin Layer Chromatography Profiling

Thin layer chromatographic analysis was performed using pre-coated silica gel and alumina glass plates. Elutions were visualized using the UV lamp and visualization agent for flavonoids as per Harborne methods (1973) and the R_f values appropriately determined and utilized for classification of eluents into fractions.

The elution solvent for thin layer chromatography comprised of methanol, chloroform and ethyl acetate in the ratio 2:1:1 v/v/v. Visualization of the TLC plates was by spraying with sulphuric acid before observing under UV lamp at 254 nm and 280 nm.

The sprayed chromatograms were dried in the oven at 105˚C then distances eluted measured for R_F values calculation in respect to the solvent front.

The fractions having similar R_F values were pooled together affording 5 fractions for the wet season and 4 fractions for the dry season at the end of the thin layer chromatography. These fractions were then concentrated through rota evaporation and stored in a freezer at temperatures below 0˚C awaiting mesquitol HPLC quantification. The respective R_f values were then calculated according to the formula below.

$$R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent}} \quad \text{...............equation 3.3}$$

3.3.3 Preparative Thin Layer Chromatography (PTLC)

The chosen semi-pure fraction, was purified further via preparative thin layer chromatography and later collected for test of boiling point and further analysis.

3.3.4 FT-IR analysis

A Fourier Transform Infrared spectrometer (FTIR) Shimadzu 8400 series was used for the analsis of different samples by taking approximately 2 mg of the resultant fractions and mixing it with about 150 milligrams of FTIR grade KBr and then grinding it into fine powder which was subsequenlty developed into a thin film for analysis. The spectra were recorded over an absorbtion range of 4000-500 cm^{-1}.

3.4 High Performance Liquid Chromatography (HPLC) Analysis

3.4.1 Mesquitol HPLC quantification method development and validation

The HPLC method was developed and validated according to ICH guidelines (ICH, 2003). Purified mesquitol (purity > 95%) was utilized as standard and all solvents (HPLC grade) re-filtered and degassed before use.

The HPLC analysis was performed on an HPLC-UV (Shimadzu LC–10AT Liquid Chromatography system with SPD–10A UV-Vis detector). The UV detector, was set at 280 nm for mesquitol maximum absorption. The mobile phase was selected by assessing different ratios of Acetonitrile: water (formic acid) ratios as 1:1, 1:4, 2:3, 3:2, and 7:3. The optimum flow rate was then investigated by interchanging the flow rate from 1 $mL.min^{-1}$, 0.8 $mL.min^{-1}$, 0.6 $mL.min^{-1}$ and 0.4 $mL.min^{-1}$. Two pressure levels were evaluated for pump pressure A and pump pressure B set at a maximum of 15.0 MPa and a minimum of 2.0 MPa. Two sets of injection volumes 10 µL and 20 µL of sample were also compared to get the best peak resolution.

Stock solution for mesquitol standard was prepared by dissolving 50 mg of mesquitol into 100 mL of acetone. The stock solutions were then serially diluted to give standard solutions of concentrations: 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL and 160 µg/mL respectively for calibration curves construction to establish linearity. The developed mesquitol HPLC quantification method was then validated by evaluation of the Regression Coefficient (R^2) for linearity check. The relative standard deviation (RSD) was used to establish precision while the accuracy was assessed by the determination of analytical recoveries which entailed intra-day and inter-day evaluations by quantifying mesquitol in three concentration levels of the set of standard solutions

exhibiting higher linearity. This was by HPLC analysis on the same day and on three consecutive days.

The limits of quantification and detection were calculated by the simultaneous analysis of mesquitol in samples and using standard deviations of pre-determined analytical recoveries. The Limit of detection and Limit of quantitation was calculated using the following equations adapted from Munyendo *et al.,* (2015).

$$\mathrm{LOD} = 3.3 \times {}^{\sigma}/_{s} \quad \text{...................... Equation 3.4}$$

$$\mathrm{LOQ} = 10 \times {}^{\sigma}/_{s} \quad \text{....................... Equation 3.5}$$

Where

σ- Standard deviation

s – Slope of the calibration curve

The robustness of the developed HPLC method was tested by altering specific experimental conditions. Effect of flow rate on peak resolution was changed gradually from 0.5 $mL.min^{-1}$, 0.75 $mL.min^{-1}$ and 1 $mL.min^{-1}$ while the mobile phase remained the same. Effect of injection volume on the resolution was also studied at 10 µL, 15 µL and 20 µL. The effect of the temperature was also studied by keeping the column thermostated at 15˚C, 25˚C and 50˚C respectively.

3.4.2 Mesquitol HPLC quantification

The developed and validated HPLC method was used to quantify the amounts of mesquitol in crude extracts and column fractions in order to establish the mesquitol abundance dynamics in the plant *P. juliflora.*

3.4.2.1 Determination of mesquitol in crude extracts

Fresh crude extracts to be used for analysis were prepared by first reconstituting a stock solution of 1mL of crude extract in 100 mL of mobile phase that consisted of acetonitrile: water (formic acid 0.1 %) in the ratio 70:30 v/v. Different dilutions were prepared by diluting 6.25 mL, 12.5 mL, 25 mL and 50 mL of this stock solution in 100 ml volumetric flask using the mobile phase to obtain samples for HPLC analysis.

A volume 20 µL of sample was then injected into the sample injection loop and the sample loaded into the C_{18} 15 cm X 2.1 mm X 2.7 µm luna^R column.

3.4.2.2 Determination of mesquitol in column chromatography fraction

Stock solutions of the column fractions were prepared by diluting 1 mL of sample in 10 mL of acetone then diluted appropriately as the case of crude extracts to obtain analysis samples. A 20 µL of sample was then injected into the sample injection loop and the sample loaded into the C_{18} 15cm X 2.1mm X 2.7µm luna^R column.

The detections were by a UV detector at λ_{max} of 280nm wavelength for quantification of mesquitol in *P. juliflora* plant samples of different ages and collected during the rain and dry seasons. The pre-developed calibration curves were applied to extrapolate the exact mesquitol abundances in milligrams.

3.5 Data Analysis

All experiments done were in triplicates. The average mean and the respective standard deviations were then calculated and thereby adequately reported as the mean values ± the standard deviations. All experimental data was presented in tables and arranged using the Microsoft Excel 2016 computer software. The data was then subjected to

statistical analysis to ascertain its validity. Analysis of variance (ANOVA) was used to establish whether variation of age and season of was significant with regards to method of extraction and solvent.

Statistical Package for Social Scientists (SPSS) version 23 was then used to establish the significance of variability between and within different groups (sample season, sample age, extraction method and solvent) with amount of mesquitol as the dependent variable to establish the significance at 95.5% level of confidence.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Solvent Extractive Value of *P. juliflora*

A tabular comparison of the variations of the percentage crude extract yields with respect to the seasons of sampling, age groups and for different methods of extraction were carried out and presented in table 4.1 below. The results showed clearly that the choice of these various solvents of different polarity played a crucial role in the percentage yields of the extracts. For all the extractions done, the soxhlet method of extraction proved to be more efficient than maceration. This was because, it produced higher yields of percentage crude extracts.

Table 4.1 below**,** shows the percentage yields of crude extracts per solvent system and extraction method.

Table 4.1: Percentage Yields of Crude Extracts per Solvent System and Extraction Method.

Sample	Solvent system	Crude extract	Percentage yields per method	
			Maceration	Soxhlet
WBl	Hexane	WBl-Hex	0.59 ± 0.15	0.86 ± 0.21
	Dichloromethane	WBl-DCM	0.89 ± 0.26	1.32 ± 0.34
	Acetone	WBl-Ace	1.02 ± 0.43	3.15 ± 0.86
	Methanol	WBl-Met	1.56 ± 0.41	4.21 ± 0.81
WAb	Hexane	WAb-Hex	0.95 ± 0.39	2.34 ± 0.54
	Dichloromethane	WAb-DCM	1.36 ± 0.48	3.68 ± 0.51
	Acetone	WAb-Ace	2.09 ± 0.86	7.69 ± 1.56
	Methanol	WAb-Met	2.76 ± 0.69	10.22 ± 2.03
DBl	Hexane	DBl-Hex	0.46 ± 0.13	0.84 ± 0.27
	Dichloromethane	DBl-DCM	0.81 ± 0.35	0.95 ± 0.41
	Acetone	DBl-Ace	0.96 ± 0.29	2.98 ± 0.49
	Methanol	DBl-Met	1.44 ± 0.42	3.16 ± 0.75
Dab	Hexane	DAb-Hex	0.89 ± 0.26	2.14 ± 0.52
	Dichloromethane	DAb-DCM	1.13 ± 0.31	3.46 ± 0.75
	Acetone	DAb-Ace	1.91 ± 0.54	7.19 ± 1.01
	Methanol	DAb-Met	2.43 ± 0.59	9.86 ± 1.21

Key: Hex: Hexane — Met- Methanol

DCM: Dicloromethane — Ace: Acetone

The extractive content of crude extracts found in *P. juliflora* agrees with other reported data from plant species found in tropical regions (Neya *et al*., 2004). Soxhlet method was henceforth adopted as the preferred extraction method for subsequent extractions.

It was also observed that, there was a direct relationship between the percentage yield of the plant crude extracts and the polarity of the extraction solvent, i.e as solvent polarity increased, the percentage yields of the extracts increased drastically. The variations in extractable components from the plant, can be accounted for by the fact that there exist different plant compounds that are of different chemical compositions and characteristics that may or may not be suitable or soluble for extraction in a specific solvent system (Fatima *et al.,* 2015).

The results also showed that the wet season, had a higher yield of crude extracts than the dry season. It was also noted that plants of the ages above 4 years produced more plant extractives than those below four years.

4.2 Flavonoids Extraction Scheme Optimization

As indicated from the crude extracts yields, the most desirable plants samples to be used for extractions were those falling in the age category of above four (4) years and sampled during the wet season. Since soxhlet was more desirable than maceration as an extraction method, optimization was performed with only samples acquired in line to these criteria.

4.2.1 Extraction solvent suitability

Solvent suitability was evaluated by investigating the yields of various solvent blends of two solvents at a time. The obtained percentage yields were as indicated in Figure

4.1 below.

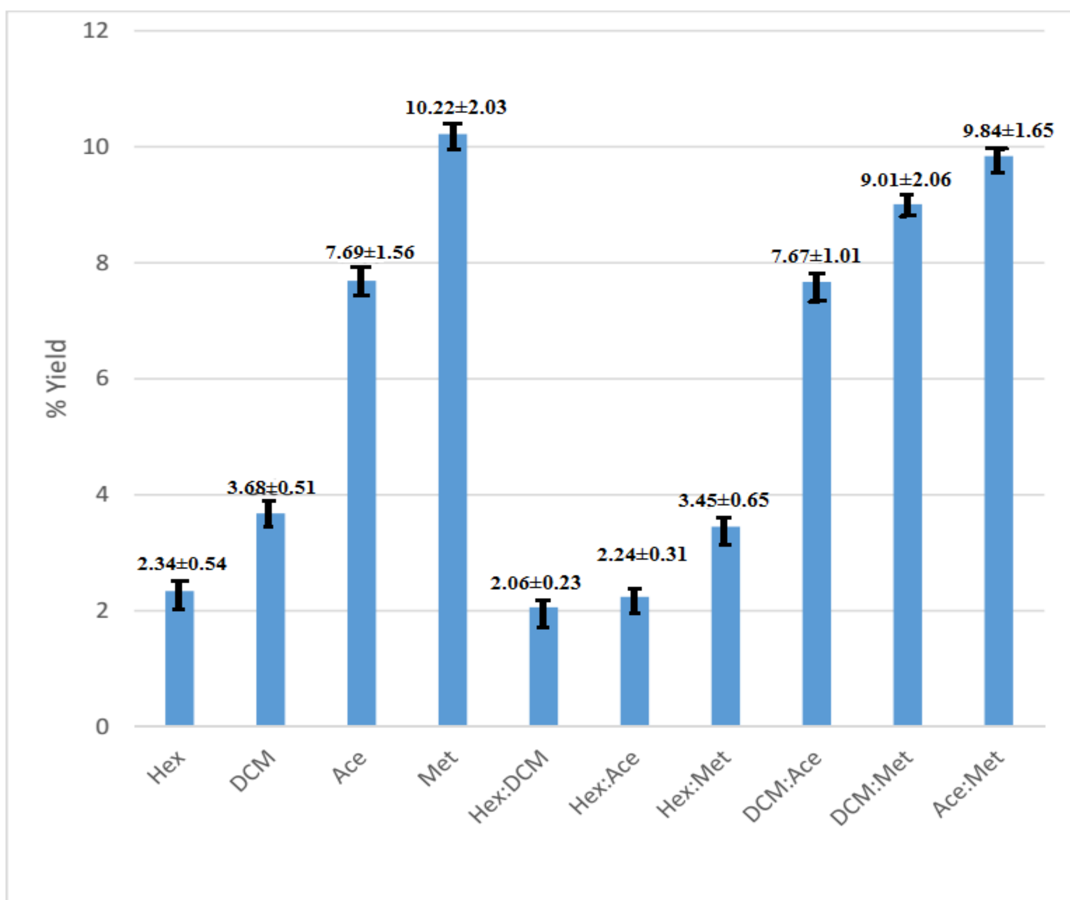


Figure 4.1: % Yield with Various Solvent Systems

Pure methanol gave the highest yield of 10.22 ± 2.03 % even above the solvent blends with the highest solvent blend being that of acetone and methanol which gave a yield of 9.79 ±1.69 %. The lowest yield amongst the blends was obtained by a blend of hexane and dichloromethane which gave 2.04 ± 0.43%. These variations can be accounted for equally by the fact that solvent polarities greatly affect the type of extractives being extracted by a plant (Fatima *et al.,* 2015).

Based on this results, it could be inferred that solvent blending did not alter the solvent suitability in extraction of extractives from *P. juliflora* heart wood of above four years in age sampled during the wet season.

4.2.2 Total Flavonoid Determination

The following calibration curve was used for the quantifying total flavonoid content in the plant extracts.

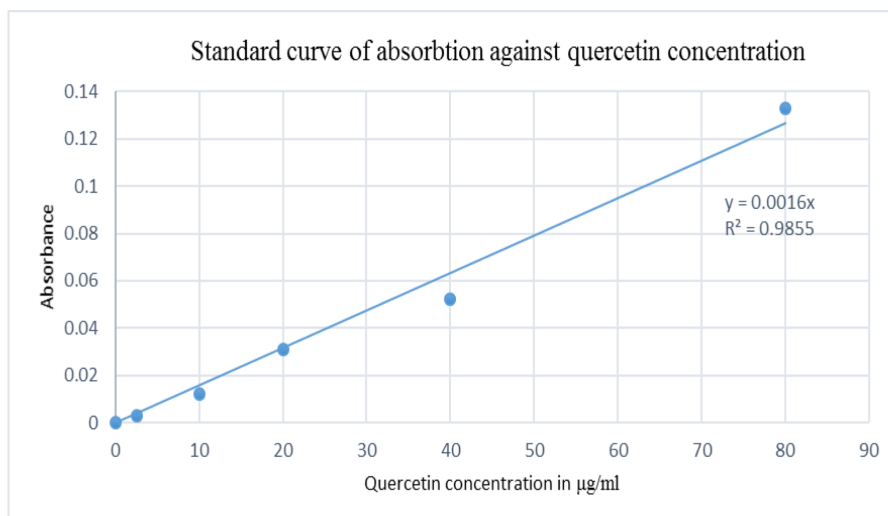


Figure 4.2: Standard Curve of Absorbance against Quercetin Concentration.

As shown in Figure 4.3, a good correlation coefficient ($r^2 = 0.9855$) was obtained from the standard curve. The total flavonoid contents (in mg of quercetin equivalent per gram dry weight) of the heartwood of *P. juliflora* for all crude extracts obtained with pure solvents and their blends are shown in Table 4.3. The highest amounts of flavonoids were found in the acetone extract (WAb-Ace) at 21.7 ± 4.93 mg/g of dry weight while the lowest were recorded in the hexane extract (WAb-Hex) that afforded 2.2 ± 0.86 mg/g.

Table 4.2: Total Flavonoid Contents for Crude Extracts

Sample	Total flavonoids (mg/g)
WAb-Hex	2.2 ± 0.86
WAb-DCM	5.5± 1.21
WAb-Ace	21.7± 4.93
WAb-Met	11.5± 2.64
WAb-Hex/DCM	4.8± 1.01
WAb-Hex/Ace	5.8± 0.97
WAb-Hex/Met	2.9± 0.75
WAb-DCM/Ace	14.5± 3.53
WAb-DCM/Met	8.6± 3.87
WAb-Ace/Met	5.9± 1.87

This finding informed the use of the crude extract, WAb-Ace, in subsequent investigations of flavonoid mesquitol abundance dynamics in the plant *P. juliflora* and for the analytical method development.

4.3 Isolation of the flavonoid mesquitol from *P. juliflora* extract

4.3.1 Column Chromatographic isolation

The Acetonic crude extracts, gave four distinct semi pure fractions for both the samples DBl-Ace and DAb-Ace (F1, F2, F3 and F4) and five distinct semi pure fractions of (F1, F2, F3, F4 and F5) for WBl-Ace and WAb-Ace. The semi pure fractions F3 were chosen and subjected to further analysis. The obtained samples, were subsequently subjected to TLC for further identification and samples with similar R_f values, were pooled together into one sample and later spotted again to ascertain their purity.

4.3.2 Preparative Thin Layer Chromatography

The semi-pure fraction F3, that was sufficient in amount, obtained at polarity gradient ratio of ethyl acetate: acetone (5:5) and on subjection to Preparative Thin Layer Chromatography (PTLC) resulted in two main streaks noted as (F3-A and F3-B).

Upon recovery with pure acetone and chilling, the steak at R_f value of 0.41 yielded yellowish crystal that were washed further with chilled acetone to crystalize out as pure cream coloured crystal. This compound was suspected to be mesquitol and was subjected to further confirmatory tests. Figure 4.3 is a PTLC of the compound mesquitol.

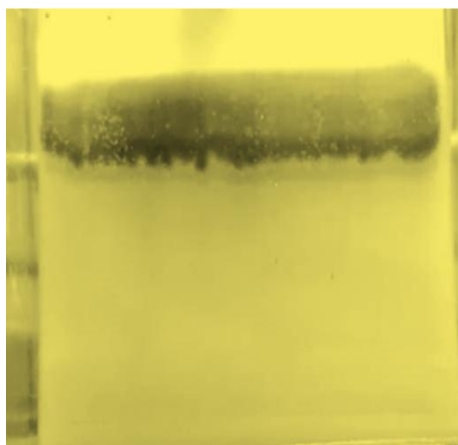


Figure 4.3: PTLC of the compound mesquitol.

4.3.3 Testing of Melting points

The melting points of the yellowish coloured compound were tested and found to be in the range of 82-84 degrees Celsius**.**

4.3.4 Mesquitol confirmation by Fourier Transform Infrared Spectroscopy

The isolated and crystalized cream coloured crystals from each of the samples were confirmed by Infrared spectroscopy to contain all the important functional groups that

are characteristic of the compound mesquitol. The resultant spectra were then compared for their similarities with mesquitol spectrum as in Figure 4.4 below.

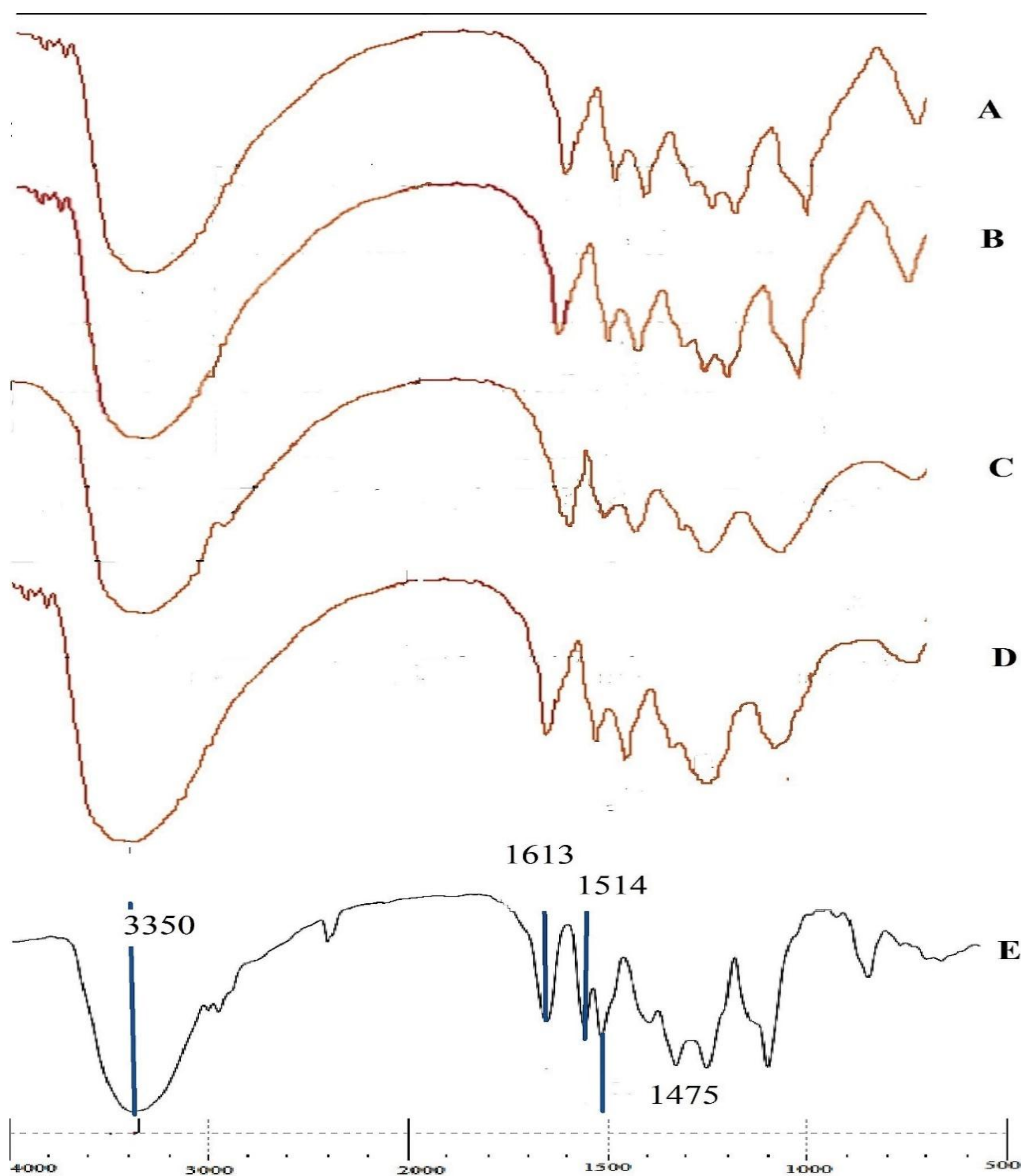


Figure 4.4: Comparison of different FTIR Spectra with that of pure mesquitol

Key A- WBl-Ace, B- WAb-Ace,

C- DBl-Ace D- DAb-Ace

E- Spectrum of pure mesquitol.

The specific identification groups for flavanols were compared as in table 4.3 below.

Table 4.3: FTIR spectra of spectra of WBl-Ace, WAb-Ace, DBl-Ace and DAb-Ace with specific interest to the functional groups for flavanols.

WBl-Ace	WAb-Ace	DBl-Ace	DAb-Ace
3448	3356	3352	3359
1612	1617	1621	1614
1522	1522	1526	1522
1489	1486	1476	1483

The resultant spectra for the samples WBl-Ace, WAb-Ace, DBl-Ace and DAb-Ace all had the following noticeable peaks that are characteristic of the flavanol structure. The C=C bond that has a skeletal vibration of around 1631 cm^{-1}, 1519 cm^{-1} and 1483 cm^{-1}. There were also noticeable peaks in the fingerprint region of around 3371 cm^{-1} indicating the presence of a hydroxyl group, a C-H bond indicated by the peaks at around 780 cm^{-1} and a C-O stretch bond indicated by a peak of around 1258 cm^{-1} and final C-H stretch of around 2950 (Muruganantham *et al.*, 2009 ; Ragavendran, *et al.,*2011).

The other stretchings were found to be relatively comparable with other IR Spectra of pure mesquitol. Hence the compound was confirmed to be mesquitol.

4.4 High Pressure Liquid Chromatography (HPLC) Analysis

4.4.1 HPLC quantification method development and validation

4.4.1.1 Optimization of chromatographic conditions

The HPLC column temperature was maintained at a constant of 25˚C with the machines UV detector maximum (λ_{max}) set at 280 nm. The mobile phase, produced the best peak resolution by using a mixture of acetonitrile: water (formic acid 0.1 %) in the ratios of 70:30 respectively.

An optimum flow rate for the HPLC system established was 0.4 mL min^{-1}, this facilitated for the reduced longitudinal diffusions through the column length, thus it gave the most effective separations with highest resolutions. Variations of the flow rate from 7.5 mL min^{-1} to 0.1 mL min^{-1} resulted in shortening of the retention time for mesquitol to an ideal pronounced peak at 3.5 minutes as the retention time with all cases having the peaks area remaining the same.

The pump pressures of the HPLC were equally varied from 8.0 MPa to a maximum of 15.0 MPa, these pressures were realized to sustain stable flow. The auto-sampling by the machine delivered a 10µm sample to the column.

The resultant chromatogram, was as in Figure 4.5.

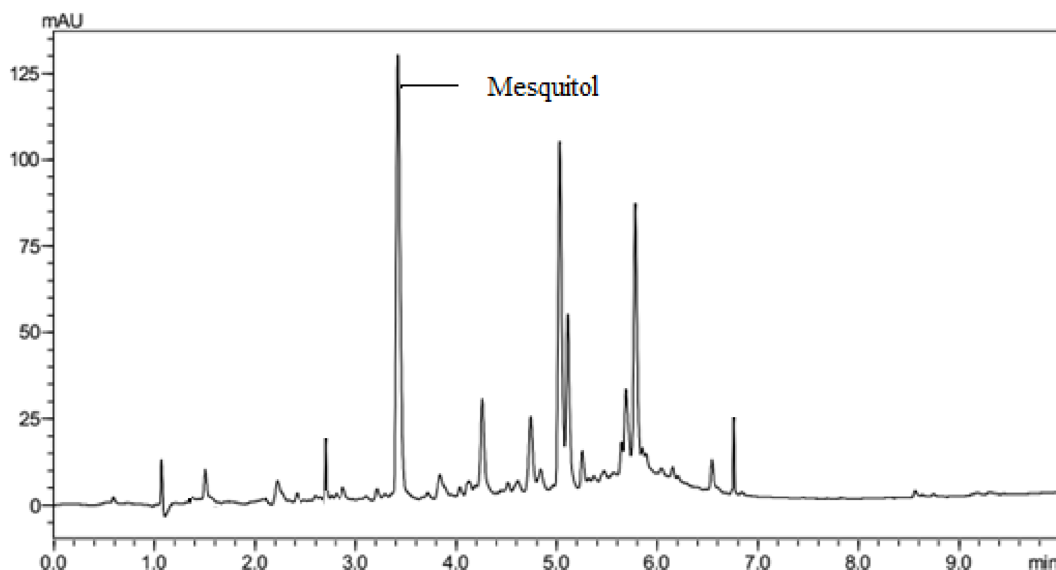


Figure 4.5: HPLC chromatogram of mesquitol

4.4.1.2 Calibration curves

The best concentration range producing the best linearity was found to be that of the ranges 10µg/mL, 20µg/mL, 40µg/mL, 80µg/mL and 160µg/mL standard solutions. This gave a straight line curve with the equation y=20885x + 7980.6 with an R^2 value of (0.9983) and the Y intercept being at 7980.

The resultant calibration curve was used to quantify the concentrations of mesquitol in the crude extracts of *P. juliflora*. The resultant data gave the calibration curve as in Figure 4.6 below.

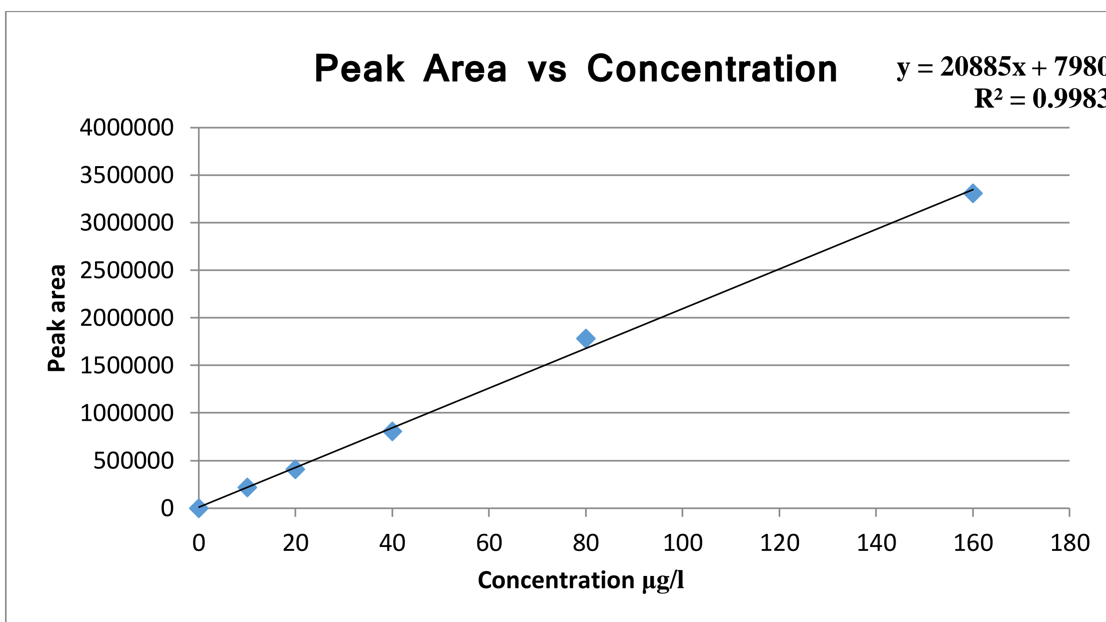


Figure 4.6: Calibration curve for mesquitol standards

4.4.1.3 HPLC method validation

4.4.1.3.1 Precision

The developed methods precision, was assessed in terms of its repeatability and intermediate precision both intraday and inter-day. The precision, was expressed in terms of percentage relative standard deviation (%RSD) and it was determined by analysing the mesquitol solution over the ranges of between 10-160 three times a day over a period of three days. The % RSD ranged from 0.0046% to 0.1095% signifying that the developed method was highly reproducible thus indicating that the variation of analysis would not be significant between different concentrations whether low or high (Kumudhavali *et al*., 2010).

4.4.1.3.2 Accuracy

The methods accuracy, was established by performing recovery experiments with the results ranging from 98.211% to 99.642% as in Table 4.4 The percentage recovery was within (98%-100%) hence the method was deemed to be accurate (Sri *et al*., 2013).

Table 4.4: Recovery results for intraday and interday analysis of mesquitol standards.

Conc. μgmL^{-1}	Day1	Day2	Day3	Mean	S. D	RSD	RSD%
10	99.301	99.242	99.642	99.395	0.21593	0.002172	0.2172
20	99.465	99.316	99.214	99.337	0.12623	0.001271	0.1271
40	98.324	98.211	98.109	98.215	0.10755	0.001095	0.1095
80	98.713	99.213	99.435	99.120	0.36981	0.003731	0.3731
160	99.514	98.937	99.147	99.199	0.29204	0.002944	0.2944
Mean	99.0634	98.9838	99.1094				
S. D	0.52208	0.455238	0.592206				
RSD	0.00527	0.004600	0.005975				
RSD%	0.52701	0.46001	0.597552				

4.4.1.3.3 Limit of detection and quantification

The limit of detection and quantification were found to be 9.18 µg/l and 27.84 µg/l respectively. They were calculated by the simultaneous analysis of mesquitol using the obtained standard deviations of the already determined analytical recoveries. The LOD, was approximately three times lower than the LOQ hence satisfying the ICH

recommendations on the relationship between the two meaning that the developed method was sensitive (Maithani *et al.,* 2010)

4.4.1.3.4 Robustness

The flow rate was first varied from 7.5mL min^{-1} to 0.1mL min^{-1} . The pump pressures of the HPLC, were then equally varied from 8.0 MPa to a maximum of 15.0 MPa. The deliberate variations of the two, showed that the method remained unaffected by the small changes of the two variables showing the methods robustness.

4.4.1.3.5 Selectivity and specificity

The methods selectivity was confirmed from the observed chromatograms with a comparison with pure mesquitol. The retentions time of the observed chromatograms of pure mesquitol and our samples were the same confirming the methods specificity.

4.4.1.3.6 Linearity

The linearity of the method, was established within the ranges 10µg -160µg/l with triplicates of the concentrations 10,20,40,80 and 160 µg/l. this resulted into the regression equation of a straight line of the form y=20885x+7980.6. The correlation coefficient (R^2) was 0.9983 showing a good correlation between the sample concentrations and the peak response areas hence showing the response is linear for the above ranges (Kumudhavali *et al*., 2010).

4.4.2 Quantification of mesquitol

Mesquitol quantification was carried out, by determining the concentration in the crude extracts through calculation from the calibration curves by the developed HPLC analysis. The data was then extrapolated back to the sampled *P. juliflora* plant material

to ascertain the abundance dynamics with season and plant age group. This was appropriately represented in Table 4.5, below:

Table 4.5: Mesquitol abundances in *P. juliflora*

Sample	Concentration of mesquitol in crude extracts (µg/mL)	%Abundance
WAb-Ace	634.162	4.943
	642.893	5.001
	615.399	4.796
WBl-Ace	209.094	1.630
	211.255	1.647
	203.659	1.587
DBl-Ace	189.367	1.473
	181.245	1.413
	190.026	1.481
DAb-Ace	533.412	4.158
	542.198	4.226
	561.426	4.376

The determined mesquitol content displayed in the Table above is in agreement with results obtained by (Azam *et al*., 2011) who found mesquitol abundance in *P. juliflora* heartwoods to be in the ranges of 4 - 7%. And Sirmah (2009) who found it to be around 8%. Our results, found mesquitol to be in the ranges of 2.9 - 5 %.

The use of the validated RP-HPLC method for the quantification of mesquitol, in *P. juliflora* was a success. It illustrated optimum and high accuracy, precision, specificity,

robustness and linearity. The method was hence substantial for satisfactory performance in the evaluation of mesquitol content from *P. juliflora*.

4.5 Statistical Data Analysis

For objective number four of the study to achieved, the three hypotheses that were previously formulated, were tested at significance level of 0.05 using SPSS 23.

Null hypothesis 1 (Ho) the amount of mesquitol in *P. juliflora* does not vary as a result of the season of harvest. A one way ANOVA was done to test the above null hypothesis. The significance of age was found to be 0.00 which is less than 0.05. this means that the hypothesis is to be rejected. Hence it could be inferred that the amount of mesquitol content in *P. juliflora* varies as a result of the season of harvest of the plant.

Table 4.6: One way ANOVA table for seasonal variation of mesquitol.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	268159.209	1	268159.209	2521.658	.000
Within Groups	425.370	4	106.342		
Total	268584.579	5			

Null hypothesis 2 (Ho) the amount of mesquitol in *P. juliflora* does not vary as a result of the age of the plant. The significance of age was found to be 0.00 which is less than 0.05 meaning that the hypothesis is to be rejected. Hence it could be inferred that the amount of mesquitol content in *P. juliflora* varies as a result of the age of the plant.

Table 4.7: One way ANOVA table for age variations of mesquitol.

	Sum of Squares	Df	Mean Square	F	Sig.
Between groups	193105.442	1	193105.442	1685.037	.000
Within Groups	458.400	4	114.600		
Total	193563.843	5			

Null hypothesis 3 (Ho) the amount of mesquitol in P. *juliflora* does not vary as a result of both the age and season of harvest.

Table 4.8: Two way ANOVA table for seasonal and age variation of the quantity of mesquitol.

Dependent Variable: concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	**469733.467[a]**	**3**	**156577.822**	**1417.363**	**.000**
Intercept	**1851923.186**	**1**	**1851923.186**	**16763.847**	**.000**
age	**3073.536**	**1**	**3073.536**	**27.822**	**.001**
season	**8468.816**	**1**	**8468.816**	**76.661**	**.000**
age * season	**458191.115**	**1**	**458191.115**	**4147.605**	**.000**
Error	**883.770**	**8**	**110.471**		
Total	**2322540.423**	**12**			
Corrected Total	**470617.237**	**11**			

a. R Squared = .998 (Adjusted R Squared = .997)

The significance of both the age and season of harvest was found to be also 0.00 which is also less than 0.05 hence it was also concluded that both the age and season of harvest together affect the amount of mesquitol present in *P.juliflora*.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

Initially, two extraction methods i.e soxhlet and maceration were used and each compared for their % yields. In all the cases, the soxhlet method of extraction proved to be more efficient and reliable as it had a relatively higher % yields. Subsequently four solvents of increasing polarity i.e hexane, dicloromethane, acetone and methanol were compared for ability to extract phytochemicals. Methanol was found to have the highest amount of percentage yields, when compared to the other three. It was also found that, the more polar the solvent was, the higher the amount of extractives extracted from the plant.

The Total flavonoid content of the heartwood of the plant showed that the acetonic extracts of plants harvested in the wet seasons were more than the others. Mesquitol was extracted and isolated from the plant majorly from the crude acetonic extracts which had the highest amount of the compound. It was also noted that blending the solvents had no significant effect on the yield of mesquitol hence soxhlet, serial extraction was recommended.

The amount of mesquitol content varied as a function both age and seasons with plants of above 4 years having higher amounts than those below the age of 4 with plants harvested in the wet season having more mesquitol content than those harvested on the dry seasons. Based on our results it can be concluded that the best harvest period for mesquitol is during the wet seasons and the plants selected should be of above 4 years.

A HPLC analytical method for the quantification of mesquitol was also developed and validated based on the ICH guidelines. The method proved to be accurate, sensitive, robust and precise hence it was recommended for use.

5.2 Recommendations

The following are recommended from this study

i) Plants of ages of about 12, 16 and 20 years of age should be examined to avail more information about mesquitol abundance trends in older plants.

ii) The relatively high levels of flavonoids in the plant suggest that the plant *P.juliflora* is a source of variant flavonoids. It is therefore recommended that more research should be done on this.

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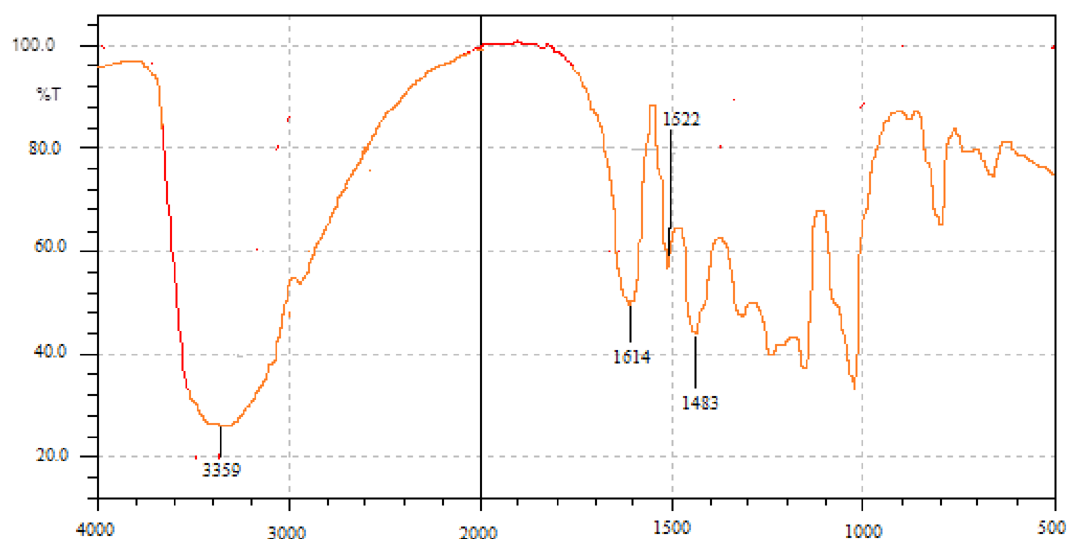
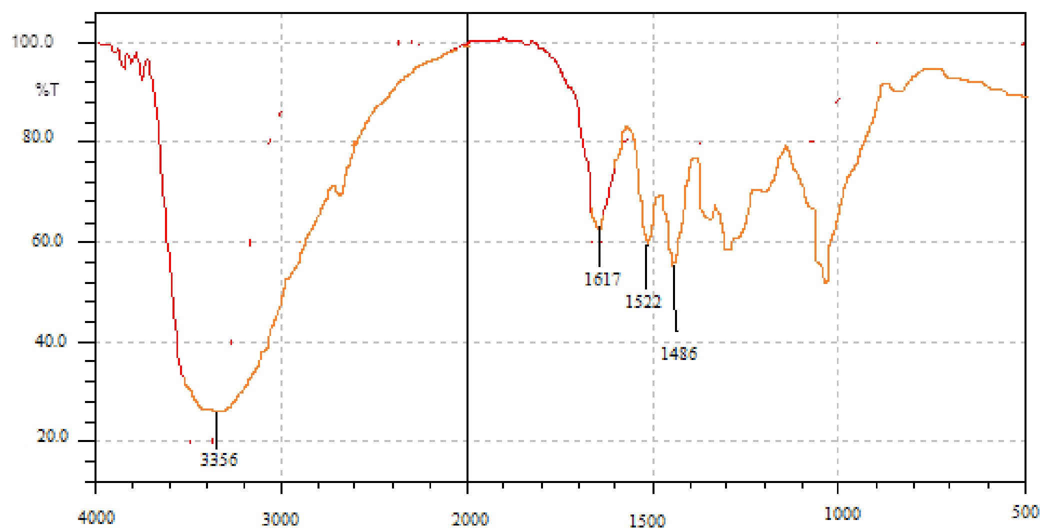
APPENDICES

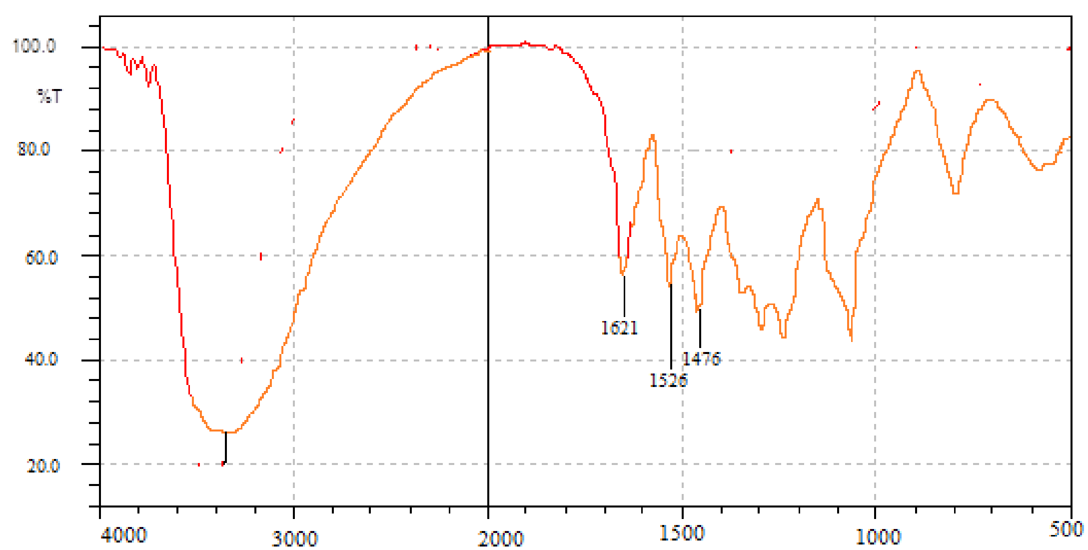
Appendix 1: FTIR Spectrum of DBl-Ace



Appendix 2: FTIR spectrum of DAb-Ac



Appendix 3: FTIR spectrum of WBl-Ace



Appendix 4: FTIR spectrum of WAb-Ace